

Mesoangioblasts of inclusion-body myositis: a twofold tool to study pathogenic mechanisms and enhance defective muscle regeneration

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Mesoangioblasts are a class of adult stem cells of mesoderm origin, potentially useful for the treatment of primitive myopathies of different etiology. Extensive *in vitro* and *in vivo* studies in animal models of muscular dystrophy have demonstrated the ability of mesoangioblast to repair skeletal muscle when injected intra-arterially. In a previous work we demonstrated that mesoangioblasts obtained from diagnostic muscle biopsies of IBM patients display a defective differentiation down skeletal muscle and this block can be corrected *in vitro* by transient MyoD transfection. We are currently investigating different pathways involved in mesoangioblasts skeletal muscle differentiation and exploring alternative stimulatory approaches not requiring extensive cell manipulation. This will allow to obtain safe, easy and efficient molecular or pharmacological modulation of pro-myogenic pathways in IBM mesoangioblasts. It is of crucial importance to identify factors (ie. cytokines, growth factors) produced by muscle or inflammatory cells and released in the surrounding milieu that are able to regulate the differentiation ability of IBM mesoangioblasts. To promote myogenic differentiation of endogenous mesoangioblasts in IBM muscle, the modulation of such target molecules selectively dysregulated would be a more handy approach to enhance muscle regeneration compared to transplantation techniques.

Studies on the biological characteristics of IBM mesoangioblasts with their aberrant differentiation behavior, the signaling pathways possibly involved in their differentiation block and the possible strategies to overcome it *in vivo*, might provide new insights to better understand the etiopathogenesis of this crippling disorder and to identify molecular targets susceptible of therapeutic modulation.

Key words: mesoangioblasts, myogenic stem cells, inclusion-body myositis, muscle regeneration

For long time, satellite cells have been considered the only myogenic source for post-natal growth, repair and maintenance of skeletal muscle. More recently several blood-born and muscle-resident stem cells have been identified in interstitial spaces of skeletal muscle with the capability to differentiate into myogenic cells, thus contributing to *de novo* formation of muscle fibers (1-4). Mesoangioblasts are a new class of adult stem cells of mesoderm origin, potentially useful for the treatment of primitive myopathies of different etiology (5). These cells, firstly isolated from dorsal aorta of murine embryos, have been largely studied in murine models, demonstrating their ability to extensively grow *in vitro*, maintaining their differentiation potential down the different mesodermal tissues (smooth and skeletal muscle, fat and bone) (6,7). In addition, mesoangioblasts are capable to form muscle fibers after direct intramuscular injection and, more importantly, intra-arterial delivery into immune deficient dystrophic α sarcoglycan (α SG) null mice. In particular, by flowing through blood circulation they migrate into downstream skeletal muscles, mainly reaching areas of muscle degeneration/regeneration, repairing skeletal muscle with concomitant recovery of global muscle function (8). The therapeutic value of mesoangioblasts in large animal models was recently demonstrated in a canine model of Duchenne muscular dystrophy (DMD) (9). Wild type (wt) or autologous mesoangioblasts transduced *in vitro* with a lentiviral vector expressing human microdystrophin transplanted intra-arterially into dystrophic dogs led to extensive reconstitution of fibers expressing dystrophin, with improvement in the contraction force and,

in many cases, preservation of walking ability. This study showed that mesoangioblasts transplantation is a safe and effective procedure in large animals opening the way for possible future clinical trials in muscular disorders.

Human mesoangioblasts

In the last years, we and others isolated the human counterpart of murine mesoangioblasts from fragments of diagnostic muscle biopsies of patients affected by inflammatory myopathies (IM) (10), DMD (11) and facioscapulo-humeral muscular dystrophy (FSHD) (12). These cells display a high proliferative rate and can be kept in culture, maintaining a normal diploid karyotype, up to 25 population doublings (PD) when large senescent cells start to appear.

Human mesoangioblasts are able to differentiate into smooth and skeletal muscle, osteoblasts or adipocytes. When co-cultured with murine myogenic cells, exposed to muscle-differentiation medium (11) or cultured in normal human myoblasts-conditioned medium, to facilitate their commitment (10), a large proportion of cells differentiate into multinucleated myotubes.

Human cells express pericytes markers (annexin V; alkaline phosphatase, ALP; desmin; α -smooth actin, α -SMA; vimentin; platelet-derived growth factor receptor β , PDGFR β), while, at variance with their murine counterpart, do not express typical endothelial markers (CD31, CD34 and VEGF receptor 2/KDR) and M-cadherin, NCAM, cytokeratins or neurofilaments. They do not constitutively express myogenic markers (MyoD, Myf5, Myogenin, Pax7). The expression of surface antigens is as follows: strongly positive for CD13 and CD44, weakly positive for CD49b, uniformly negative, among others, for CD31, CD34, CD45, CD133 (10, 11). Together, these markers identify human adult mesoangioblasts as the *in vitro* progeny of pericytes.

Mesoangioblasts from inflammatory myopathies

In our first study, we isolated with high efficiency and characterized mesoangioblasts from diagnostic muscle biopsies of patients with idiopathic inflammatory myopathies (dermatomyositis, DM, polymyositis, PM and inclusion-body myositis, IBM). Mesoangioblasts from DM, PM and IBM retain the same proliferation ability and cell cycle distribution of cells isolated from normal muscle, and can be grown *in vitro* and expanded for as many as 25-30 passages (21.3 ± 3.21 PD), though not indefinitely. The exposure of DM and PM mesoangioblasts to normal myoblast-conditioned medium is greatly effective in inducing skeletal muscle differentiation, outlining the importance of muscle-secreted factors for myogenic maturation of these stem cells. By contrast, IBM mesoan-

gioblasts display a marked and selective impairment of skeletal muscle differentiation, with the formation of only sparse mononucleated myosin-positive myotubes under the same culture conditions promoting massive skeletal muscle differentiation of mesoangioblasts from DM, PM, and normal muscle. Of note, normal mesoangioblasts exposed to IBM-myoblast-conditioned medium efficiently differentiate down skeletal muscle.

Interestingly, cultures of myogenic (satellite-derived) MyoD-positive cells obtained from the same biopsy samples undergo, under appropriate conditions, terminal differentiation, indicating that the myogenic differentiation process can reach terminal stage in cultured satellite cells from IBM. This is in agreement with previous studies showing that cultured IBM myogenic cells proliferate and terminally differentiate and can be properly innervated (13, 14).

Defective myogenic differentiation of IBM mesoangioblasts

We showed for the first time, that a progenitor cell, resident in a perivascular niche of IBM muscle is defective in myogenic determination and differentiation. No significant differences of age existed between the IBM and DM/PM patients in our study, excluding a mere consequence of muscle aging, but strongly suggesting a causal correlation with the specific pathophysiology of IBM. Interestingly, IBM muscle, that is characterized by an inadequate long term regeneration despite a normal number of satellite cells at least early in the disease, shows scarcity in muscle connective tissue of ALP-positive cells, likely activated pericytes (representing the cells from which mesoangioblasts are established *in vitro*), and a failure of the isolated mesoangioblasts to differentiate *in vitro*. Genome wide analysis of IBM mesoangioblasts showed that, differently from their normal or other myopathies counterparts, they express high levels of transforming growth factor β 1 (TGF β 1), a known inhibitor of myogenesis (15), SFRP (Soluble Frizzled Related Protein) 2, a Wnt antagonist shown to block myogenic conversion of CD45+ SP cells (16), and BHLH (basic helix loop helix) B3, a transcription factor that inhibits MyoD activity (17), any of which might be responsible for the differentiation block. Unraveling the molecular basis of such block will likely provide both significant insights into the mechanisms of IBM muscle diminished regenerative potential involving satellite and other muscle precursor cells, as well as more selective modulatory strategies to correct the defective myogenic maturation of IBM mesoangioblasts. However, we demonstrated that MyoD transient transduction is sufficient to induce a normal progression of IBM mesoangioblasts along the skeletal muscle differentiation path.

Mesoangioblasts and inflammation in vivo

Analysis of molecular phenotype of human mesoangioblasts from IM, although with specific differences in gene expression profiles between IBM and DM, shows a general up-regulation of several inflammation-related genes (10). This probably reflects a “conditioning” effect of the local muscle environment in immune-mediated myopathies characterized by marked increase of adhesion molecules, chemokines and pro-inflammatory Th1 cytokines. Mesoangioblasts expression of adhesion molecules involved in the processes of leukocytes rolling, adhesion to vascular endothelium and transmigration across blood vessels well correlates with their ability, at variance with satellite cells, to migrate and home into damaged muscle by using their adhesion properties to reach interstitial spaces of muscle when injected intra-arterially (8). Molecules such as integrins and selectins are highly expressed during muscle inflammation, as well as secreted factors such as stromal derived factor 1 (SDF-1) and tumor necrosis factor α (TNF α) that have also a remarkable effect in improving homing to skeletal muscle of normal mouse and human mesoangioblasts (18).

IBM defective regeneration

Post-natal mesoangioblasts considered as part of the pericyte population are located in perithelial position and express ALP *in vivo* (11). It is interesting to note that a characteristic histochemical feature of PM and DM is the strong ALP-positivity in perimysial and endomysial connective tissue (normally connective tissue is ALP-negative and only blood vessels' wall and occasional regenerant muscle fibers are stained), as opposed to IBM as well as other myopathies with increased connective tissue such as DMD, usually displaying no or very little ALP-positivity (19). It has been noted that this feature correlates more with the regenerative properties of muscle rather than with inflammatory changes. In fact, ALP staining, usually negative earlier in the course of PM, persists in spite of immunosuppressive treatment that can rapidly eliminate inflammatory cells from the biopsy specimens, leading to the hypothesis that it could reflect activated or proliferating fibroblasts (19). Although ALP activity is generally considered a marker of osteoblasts differentiation, we showed data strongly suggesting that human ALP-positive mesoangioblasts likely represent an activated cell population found in the muscle connective tissue of IM, originating from the perivascular niche, susceptible of myogenic determination *in vivo*, as indicated by MyoD expression and contributing to muscle repair and regeneration. To what extent mesoangioblasts contribute to muscle regeneration, either directly or by feeding the satellite cells pool, is unknown. However,

it is conceivable that during extensive muscle regeneration, i.e. following inflammatory muscle damage as in DM-PM, activated mesoangioblasts may play a much more significant role than in normal muscle repair. On the contrary, in IBM muscle in spite of the presence of some degree of chronic lymphomonocytic inflammation, defective mesoangioblasts cannot keep up with progressive muscle fibers degeneration participating to formation of new muscle fibers.

Ongoing studies

Our previous studies have shown that adenoviral-mediated overexpression of MyoD or silencing the inhibitor BHLHB3 gene by siRNA are able to restore the progression down the myogenic pathway of IBM mesoangioblasts. However, though experimentally effective, these procedures are unlikely to be used in clinical practice as an induction treatment for autologous mesoangioblasts expanded in culture before a possible intra-arterial delivery in IBM patients. Therefore we are currently investigating different pathways involved in skeletal muscle differentiation of these cells and exploring alternative stimulatory approaches not requiring extensive cell manipulation, in order to obtain safe, easy and efficient molecular or pharmacological modulation of pro-myogenic pathways in IBM mesoangioblasts (20, 21).

Among the possible *ex vivo* treatments, we have explored *in vitro* the effects of different treatments with molecules known to act on pro-myogenic pathways, such as deacetylase inhibitors (DI, trichostatin A, TSA and 5-azacytidine) as single agents or in combination with insulin-like growth factor 1 (IGF-1) and arginine-vasopressin (AVP) that may enhance the response of IBM mesoangioblasts to DI. Transient exposure to DI or AVP followed by our differentiation protocol led to a significant increase of myotubes formation, however, the efficiency of these pharmacological approaches is not yet as good as that experimentally observed with MyoD overexpression (unpublished results). This implies that in order to become of clinical significance these treatments must be associated to others *ex vivo* approaches.

More recently, we have investigated the expression of the Rho family guanine nucleotide exchange factor (GEFT) known to be transcriptionally up-regulated during myogenic differentiation, promoting, when overexpressed by gene transfer, skeletal muscle regeneration *in vivo* through the Rho-signaling cascade (22-25). In several independent experiments, we have observed that IBM mesoangioblasts express significantly lower levels of the protein compared to normal or PM and DM mesoangioblasts. By overexpressing GEFT in IBM mesoangioblasts, using a pCMV-Tag 2B expression vector containing the

human GEFT cDNA sequence, upon exposure to differentiating medium cells were able to fuse into multinucleated myosin-positive myotubes, although with low efficiency. To determine whether IBM hGEFT-transduced mesoangioblasts kept their myogenic potential also *in vivo*, we transplanted them into the tibialis anterior of SCID mice and evaluated their ability to participate in muscle regeneration. From our *in vivo* experiments, we observed that after transplantation of GEFT-transfected IBM mesoangioblasts, many areas of injected muscle were reconstituted with fibers expressing human spectrin and containing human nuclei. Our data obtained so far, would suggest a possible functional role of GEFT in IBM muscle (manuscript in preparation).

Conclusions and future perspectives

Despite the presence also in IBM of CD8-mediated myocytotoxicity that is known to play a major role in PM, the exact pathogenic significance of inflammatory changes in this disorder is unclear, as patients respond poorly or not at all to immunosuppressive therapies. In PM and in DM, the latter being a complement-mediated microangiopathy of skeletal muscle, current immunotherapies are usually effective and suppression of lymphomonocytic infiltration of muscle obtained by steroids and immune suppressive drugs is followed by efficient muscle regeneration and recovery of muscle strength and trophism. On the contrary, IBM muscle is characterized by the presence of unique degenerative features and inefficient regenerative properties. Thus, IBM invariably progresses leading to a significant disability. Our studies showing that also from IBM it is possible to isolate cells with a high myogenic potential, such as mesoangioblasts, localized in the perivascular niche and normally not actively producing skeletal muscle, might open new therapeutic strategies of clinical relevance. However, since mesoangioblasts isolated from IBM muscle fail to normally differentiate into skeletal muscle, in order to envisage a possible clinical use of autologous mesoangioblasts as muscle regenerative cell therapy, it will be essential to stimulate *in vitro* IBM mesoangioblasts to enhance their defective myogenic differentiation. Even more important would be to successfully activate *in vivo* the endogenous mesoangioblasts present in IBM muscle inducing them to make new regenerating fibers thus actively counteracting progressive muscle degeneration. To this end, it is of paramount importance the identification of factors (ie. cytokines, growth factors) produced by muscle or inflammatory cells and released in the surrounding milieu able to regulate the differentiation ability of IBM mesoangioblasts. Modulation of such target molecules selectively dysregu-

lated in IBM muscle to promote myogenic differentiation of endogenous mesoangioblasts appears a more handy approach to enhance muscle regeneration compared to transplantation techniques.

Actually, the use of myogenic stem cells to cure any muscle disorder represents a very difficult challenge and it is now unpredictable whether it will ever come true. However, their safety as therapeutic tool has been demonstrated (9) and a phase I clinical trial with donor-derived mesoangioblasts is ongoing in DMD patients (26). Nevertheless, major technical problems exist that is necessary to overcome to achieve satisfactory transplantation and engraftment of homogeneous population of myogenic precursors. On one side, in fact, in genetic myopathies it must be demonstrated that muscle reconstitution with fibers expressing the defective gene will be clinically relevant to thwart progressive muscle weakness and degeneration. On the other side, in acquired diseases of muscle, transplanted stem cells might in turn become target of the same noxae causing the disease, thus frustrating the attempt to significantly contribute to muscle regeneration and counteract the progressive atrophy of treated muscles. Nevertheless, studies on the biological characteristics of IBM mesoangioblasts with their aberrant differentiation behavior, the signaling pathways possibly involved in their differentiation block and the possible strategies to overcome it *in vivo*, might provide new insights to better understand the etiopathogenesis of this crippling disorder and to identify molecular targets susceptible of therapeutic modulation.

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